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13. ABSTRACT (Maximum 200 Words)

The objective of the current proposal is to perform immunotherapy to eradicate prostate cancer and at the same time to avoid the development of autoimmune disease. The proposal contains two tasks. The first task is a combination of IL-2 based tumor-reactive T cell adoptive therapy with the TGF-beta based gene therapy for the treatment of mouse prostate cancer. The second task is A tetracycline inducible TGF-beta based gene therapy. At the time of this report, we have completed Task 1 and a paper has been accepted by Cancer Research for publication in its March 1, 2005, issue. Currently, we are in the process of conducting studies described in Task 2.

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INTRODUCTION:

During the funding period, we have completed studies described in Task 1. Currently, studies described in Task 2 are underway. Briefly, the progress can be summarized by a paper that have been accepted for publication in Cancer Research. In that paper, we reported the successful eradication of mouse prostate cancer by adoptive transfer of tumor-reactive TGF-beta insensitive CD8+ T cells in tumor bearing mice.

BODY:

Task 1. To perform a combination of IL-2 based tumor-reactive TGF-beta insensitive CD8+ T cells in an adoptive therapy of tumor bearing mice (see publication by Zhang et al, 2005)

* We have successfully isolated tumor-reactive CD8+ T cells from donor mice by vaccinating these mice with irradiated TRAMP-C2 mouse prostate cancer cells. The spleen of these vaccinated mice was isolated and CD8+ T cells were isolated.

* The isolated CD8+ T cells were expanded ex vivo in the presence of lysates of TRAMP-C2 cells, irradiated spleen cells (as the antigen presenting cells), IL-2, and antiCD3 antibody.

* We have inserted a dominant negative TGF-β type II receptor (dnTβRII) expression vector MSC retrovirus gene into the above mentioned tumor reactive CD8+ T cells. Under a separate start site, a green fluorescent protein expressed vector was inserted into the same retrovirus gene.

* We have successfully transferred the tumor-reactive TGF-beta insensitive CD8+ T cells to the tumor-bearing male C57BL/6 mice.

* Tumor cells were injected into above recipient mice at 21 days prior to adoptive transfer of CD8+ T cells. At 40 days after the adoptive transfer, animals were sacrificed for inspection of the status of metastasis of TRAMP-C2 mouse prostate cancer cells into the lung.

* In mice received naïve CD8+ T cells, the majority of the tumors were not rejected. In animals received tumor-reactive control CD8+ T cells, there was a partial tumor rejection. However, in animals received adoptive transfer of tumor-reactive TGF-beta insensitive CD8+ T cells, most of the tumors were rejected with no apparent toxicity.

Task 2: In progress.

KEY RESEARCH ACCOMPLISHMENTS:

- We have developed an immunotherapy approach in which we perform adoptive transfer of tumor-reactive TGF-beta insensitive CD8+ T cells into tumor bearing mice. These animals were able

to reject established mouse prostate tumors metastasized into the lung.

REPORTABLE OUTCOMES:

As a result of this research funded by the Department of Defense, we have completed a manuscript. (see appendix).

CONCLUSIONS:

Immunotherapy using adoptive transfer of immune cells is a promising approach for treating cancer patients. The presence of tumor infiltrating lymphocytes (TIL) in the tumor parenchyma has been recognized for three decades[1]. TIL were isolated from surgical specimens, clonally expanded by ex vivo culture, and adoptively transferred to the cancer patients with variable results[2, 3]. Recently, Yee and coworkers selected antigen specific $CD8^+$ T cells for ex vivo expansion and transferred these cells into patients. These $CD8^+$ T cells did not persist, requiring repeated transfer of $CD8^+$ T cells in order to elicit responses from the patients[4]. Rosenberg and colleagues treated autologous TIL cells with IL-2 for ex vivo expansion and then transferred them to patients. Again, in order for these cells to "engraft", lymphodepletion was necessary[5]. These results, although impressive, fall short of our expectation, i.e., total elimination of tumor cells in most patients.

One of the reasons for the failure of TIL in treating cancer is perhaps the fact that the functional role of TIL in cancer has been a subject of controversy[6]. Initially, it appeared that the presence of TIL in the tumor could correlate to prognosis[7]. Subsequent studies showed that TIL were functionally impaired [8-10]. This impairment of TIL has been attributed to effects exerted by the tumor microenvironment[11]. When developing an immune-based strategy for cancer therapy, in addition to immune stimulation, the issue of overcoming tumor-derived immune suppression must be taken into consideration[12]. There are 6 potential mechanisms of tumor immune escape: loss of tumor antigen expression; variations in tumor antigen; defects in the transporter associated with antigen presentation; defects in expression of the MHC heavy chain; expression of immunoprotective molecules and release of molecules by tumor cells that disrupt T-cell signaling or induce T-cell death; and upregulation of the expression of immunoprotective molecules (PI9, FLIP and the IAP family) that counteract the effects of FASL, granzyme B, CXCL12, FLIP, FLICE, IAP, IL-10, PI9, RCAS1 and TGF-β. Among these molecules, TGF-β is an important immunosuppressant[13].

TGF-β has been recognized as a potent immunosuppressive factor[14-18]. The high levels of TGF-β produced by cancer cells have a negative effect on surrounding cells, including the host immune cells and have been implicated to play a role in tumor escape from immune surveillance [19,20]. Besides the tumor, the immune system, in response to the presence of tumor, is also

able to produce a significant amount of TGF-β to down regulate immune surveillance[21].

In light of the above discussion, TGF-β appears to be an attractive target for anti-cancer therapy. Attempts to take advantage of the properties of TGF-β for the treatment of cancer have been reported. Gorelik and Flavell first described the immune-mediated eradication of tumors through the blockade of TGF-β signaling in T cells[17]. These investigators used transgenic mice with TGF-β null expression targeted specifically to T cells. Subsequently, our study using transplant of TGF-β-insensitive bone marrow cells also demonstrated a total rejection of metastatic tumor cells[22, 23]. However, in both studies, due to the non-specific nature of the immune cells, autoimmune disease eventually developed in the hosts. Results of our recent study have shown that adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells were able to eradicate established lung metastasis of mouse prostate cancer cells, TRAMP-C2[24]. In the present study, we continue to employ this novel gene therapy approach to investigate the ability these $CD8^+$ T cells to infiltration into the tumor parenchyma. Here, we report that these $CD8^+$ T cells show a distinct ability to infiltrate into established tumors, secrete relevant cytokines, and induce apoptosis of tumor cells.

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APPENDICES:

Zhang Q, Yang X, Pins M, Liu V, Javonovic B, Kuzel T, Kim S-J, Van Parijs L, Greenberg NM, Guo Y, Lee C. (2005) Adoptive transfer of tumor reactive TGF-β insensitive $CD8^+$ T cells: Eradication of autologous mouse prostate cancer. Cancer Research (In Press)

Research Article

Adoptive Transfer of Tumor-Reactive Transforming Growth Factor-β–Insensitive CD8$^+$ T cells: Eradication of Autologous Mouse Prostate Cancer

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Abstract

Transforming growth factor (TGF)-β is a potent immunosuppressant. Overproduction of TGF-β by tumor cells may lead to tumor evasion from the host immune surveillance and tumor progression. The present study was conducted to develop a treatment strategy through adoptive transfer of tumor-reactive TGF-β-insensitive CD8$^+$ T cells. The mouse TRAMP-C2 prostate cancer cells produced large amounts of TGF-β1 and were used as an experimental model. C57BL/6 mice were primed with irradiated TRAMP-C2 cells. CD8$^+$ T cells were isolated from the spleen of primed animals, were expanded *ex vivo*, and were rendered TGF-β insensitive by infecting with a retrovirus containing dominant-negative TGF-β type II receptor. Results of *in vitro* cytotoxic assay revealed that these CD8$^+$ T cells showed a specific and robust tumor-killing activity against TRAMP-C2 cells but were ineffective against an irrelevant tumor line, B16-F10. To determine the *in vivo* antitumor activity, recipient mice were challenged with a single injection of TRAMP-C2 cells for a period up to 21 days before adoptive transfer of CD8$^+$ T cells was done. Pulmonary metastasis was either eliminated or significantly reduced in the group receiving adoptive transfer of tumor-reactive TGF-β-insensitive CD8$^+$ T cells. Results of immunofluorescent studies showed that only tumor-reactive TGF-β-insensitive CD8$^+$ T cells were able to infiltrate into the tumor and mediate apoptosis in tumor cells. Furthermore, transferred tumor-reactive TGF-β-insensitive CD8$^+$ T cells were able to persist in tumor-bearing hosts but declined in tumor-free animals. These results suggest that adoptive transfer of tumor-reactive TGF-β-insensitive CD8$^+$ T cells may warrant consideration for cancer therapy. (Cancer Res 2005; 65(5): 1-9)

Introduction

Adoptive therapy using antigen-specific immune cells from patients has become an attractive approach for tumor immunotherapy (1). The procedure has been effective in cases of low tumor burden. Increased knowledge of immunoregulation and immune function of effector cells has led to the development of specific cell therapy with the objective of targeting tumor cells for destruction. The ultimate effector cell that facilitates tumor rejection in preclinical animal models is CD8$^+$ T cells (1).

Historically, adoptive transfer of lymphokine-activated killer cells was first attempted, in which *ex vivo* culture of autologous lymphocytes with interleukin 2 (IL-2) to increase the number of activated effector cells. However, a clinical benefit was not shown by this approach (2). Subsequently, tumor-infiltrating lymphocytes were isolated from surgical specimens, clonally expanded by *ex vivo* culture with IL-2, and adoptively transferred to the patient (3). Although the initial results were promising, results of a phase III randomized trial using CD8$^+$ tumor-infiltrating lymphocytes in combination with IL-2 failed to show an improved response in patients with metastatic renal cell carcinoma (4). Two recent studies represent the state-of-the-art strategies in adoptive therapy for cancer. In the first study (5), authors selected antigen-specific CD8$^+$ T cells for *ex vivo* expansion and transfer into patients. Responses were remarkable but these CD8$^+$ T cells did not persist, requiring repeated transfer of CD8$^+$ T cells to elicit responses from the patients. In the second study (6), authors treated autologous tumor-infiltrating lymphocyte cells with IL-2 for *ex vivo* expansion and then transferred to patients following lymphodepletion. Although long-term engraftment was achieved, only 4 of 35 patients showed complete response. These results, although impressive, fall short of our expectation (i.e., total elimination of tumor cells in most patients).

In considering tumor immunotherapy, the issue of tumor-derived immune suppression must be taken into consideration (1). It seems that despite the ability to generate immune cells reactive against cancer antigens, tumor escape mechanisms can overpower these immune reactions with an eventual tumor progression (7). Tumor cells have acquired many mechanisms to evade the host immune surveillance (8, 9). One of such possibilities has been the down-regulation of tumor antigen processing (10). Tumor-specific CD4$^+$CD25$^+$ T regulatory cells can also inhibit CD8$^+$ T-cell function (11). Tumor-derived immunosuppressive cytokines, including vascular endothelial growth factor, IL-10, and transforming growth factor (TGF)-β (9, 12–14), also contribute to tumor evasion of the host immune surveillance. In the present study, we propose to focus on TGF-β-mediated evasion of immune surveillance.

Tumor cells secrete large amounts of TGF-β. High levels of TGF-β produced by cancer cells have a negative impact on surrounding cells, including the host immune cells (15). TGF-β is a potent tumor-induced immunosuppressant (8, 12, 16–20). Therefore, TGF-β seems to be an attractive target for anticancer therapy. The first piece of work describing immune-mediated eradication of tumors through the blockade of TGF-β signaling in T cells was reported by Gorelik and Flavell (17). These authors used transgenic mice with

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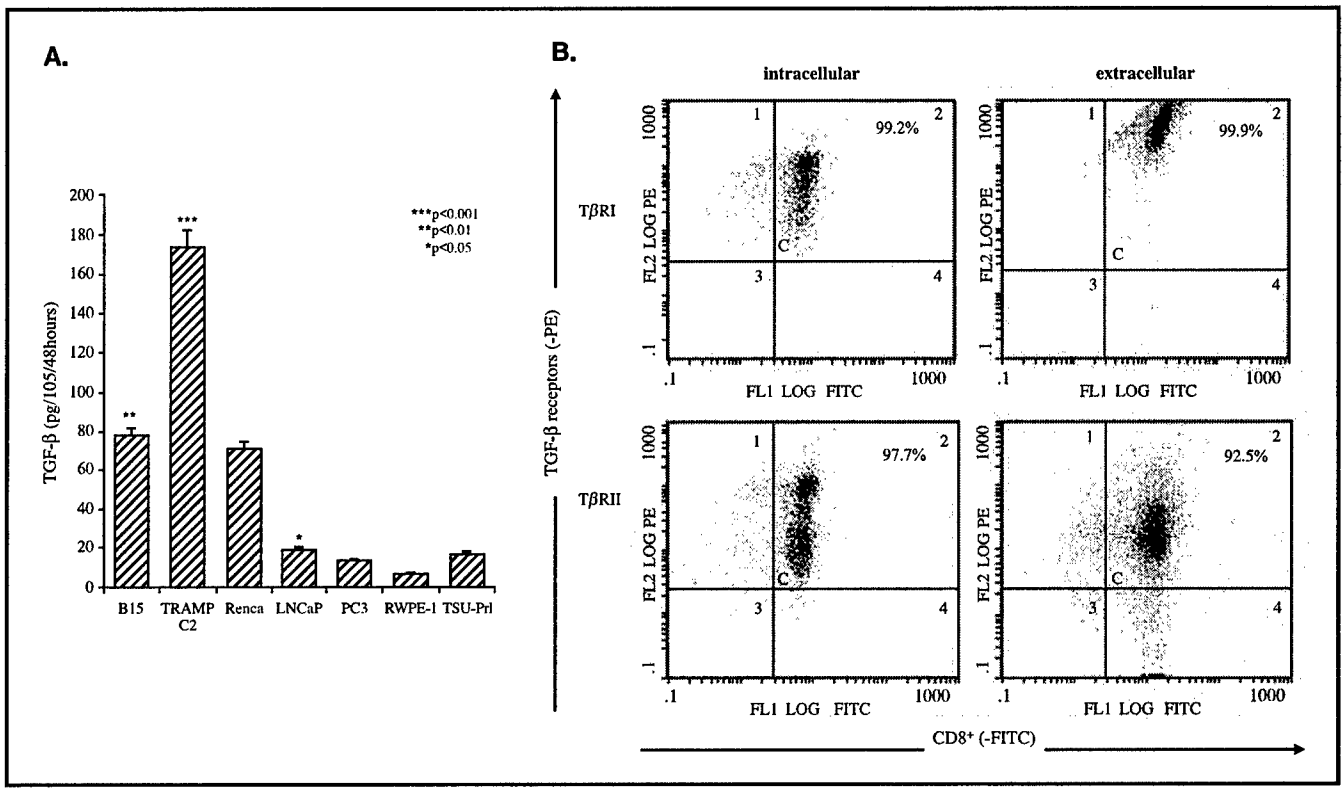


Figure 1. *A*, secretion of TGF-β1 levels by different cell lines. TGF-β1 present in the conditioned media over a period of 48 hours was determined by ELISA (see Materials and Methods) and was expressed as pg per 10^5 cells per 48 hours. TRAMP-C2, mouse prostate cancer cell line; B16, mouse melanoma cell line; Renca, mouse renal cell carcinoma cell line; LNCaP and PC3, human prostate cancer cell lines; TSU-Pr1, human bladder cancer cell line, RWPE-1, immortalized human normal prostate epithelium cell line. *Bars*, SD. *B*, expression of TGF-β type I (*TβRI*) and type II receptors (*TβRII*) in normal mouse splenic $CD8^+$ T cells. Analysis was done by double-labeled immunofluorescent FACS ($CD8^+$-FITC, TGF-β receptors-phycoerytherin; see Materials and Methods).

TGF-β null expression targeted specifically to T cells. Subsequently, our study using transplant of TGF-β-insensitive bone marrow cells also showed a total rejection of metastatic tumor cells (19, 20). However, in both studies, due to the nonspecific nature of the immune cells, autoimmune disease eventually developed in the hosts.

In the present study, we attempted to combine the above two advanced technologies by employing adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells into tumor-bearing mice. Here, we report that these $CD8^+$ T cells showed a robust antitumor activity with little or no apparent toxicity.

Materials and Methods

Mice and Cells. Male C57BL/6 mice ages 6 to 8 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University's Feinberg School of Medicine in accordance with established guidelines of the Animal Care and Use Committee of Q2 Northwestern University. TRAMP-C2 cells obtained from Dr. N. Greenberg were maintained in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin.

Generation of Tumor-reactive $CD8^+$ T Cells. Mice were primed with irradiated TRAMP-C2 cells (5×10^6 per mice at 20,000 Ci) by s.c. injection every 10 days for a total of three inoculations. Two weeks following the last vaccination, splenic $CD8^+$ T cells were isolated by using murine T cell $CD8^+$subset column kit (R&D Systems, Minneapolis, MN) and were expanded (10^5/mL) in the presence of TRAMP-C2 lysates (1×10^6) and irradiated autologous splenocytes (1×10^6/mL at 3,000 Ci) in medium containing RPMI 1640 with 10% fetal bovine serum, IL-2 (50 units/mL), anti-$CD3^+$ monoclonal antibody (30 ng/mL, R&D Systems), HEPE (25 mmol/L), L-glutamine (4 mmol/L), and 2-ME (25 mmol/L). Media were changed every 3 days.

Infection with Retrovirus Containing TGF-β Type II Receptor or Green Fluorescent Protein. $CD8^+$ T cells were cultured for at least 10 weeks before they were infected with the murine stem cell virus retrovirus containing the dominant-negative TGF-β type II receptor (TβRIIDN) and green fluorescent protein (GFP; Fig. 2*A*; refs. 19, 20). The infection efficiency was 93.9% for the TβRIIDN vector and 92.8% for the GFP control vector (Fig. 2*B*). There were three types of $CD8^+$ T cells in each time group. The first type was tumor-reactive TGF-β-insensitive $CD8^+$ T cells (tumor-reactive $CD8^+$ T cells infected with the virus containing TβRIIDN). The second type was tumor-reactive $CD8^+$ T cells infected with the virus containing the GFP control vector. The third type was naive $CD8^+$ T cells, which were freshly isolated from the spleen of naive donor animals without any treatment.

***In vitro* CTL Assay.** The above three types of $CD8^+$ T cells were subjected to a standard ^{51}Cr release assay (20). TRAMP-C2 cells were used as targets. An irrelevant cancer cell line, mouse melanoma cell line, B16-F10, was used as a nonspecific control. Target cells were labeled with 0.1 mCi of ^{51}Cr per 10^6 cells for 4 hours at 37°C, followed by 5 washes in HS-4 media and were seeded in 96-well U-bottomed plates (5,000 cells/well). $CD8^+$ T cells were added at different effector/target ratios (1:1 to 100:1) for 5 hours. The supernatants were harvested using the Skatron filters (Skatron Instruments, Sterling, VA) and the released radioactivity was measured using a gamma counter (LKB Wallac, Turku, Finland). The percent of specific lysis was determined as 100 × ([Experimental ^{51}Cr Release − Spontaneous ^{51}Cr Release]/[Maximum ^{51}Cr Release −

Spontaneous ^{51}Cr Release]). The maximum release was determined by adding 2% SDS to target cells.

In vivo **Antitumor Assay.** Mice received a single injection of 5×10^5 TRAMP-C2 cells via the tail vein. Adoptive transfer of $CD8^+$ T cells (2×10^6) was done on either day 3, 7, or 21 following tumor cell injection. Mice were maintained on antibiotics (sulfamethoxazole-trimethoprim) for a minimum of 2 weeks to prevent opportunistic infection after the injection. Forty days after the adoptive transfer, all animals were sacrificed. Some animals were sacrificed sooner than 40 days due to poor health conditions. Serum levels of IFN-γ and IL-2 were determined by ELISA. Splenic $CD8^+$ T cells were isolated, and the percentage of GFP-positive $CD8^+$ T cells in each spleen was calculated following analysis by flow cytometry.

Histologic Procedures. For each animal, upon euthanasia, the lung was excised, fixed in formalin, embedded in paraffin, and serially sectioned at 4-nm thickness until the block was exhausted. Routine H&E staining was done at an interval of every 10 sections. The unstained paraffin sections were used for studies described below.

Nuclear Staining, Apoptosis Assay, and $CD8^+$ Staining. Tissue sections were subjected to apoptosis assay by using the TUNEL apoptosis kit (Upstate, Lake Placid, NY) and were labeled with Avidin-FITC

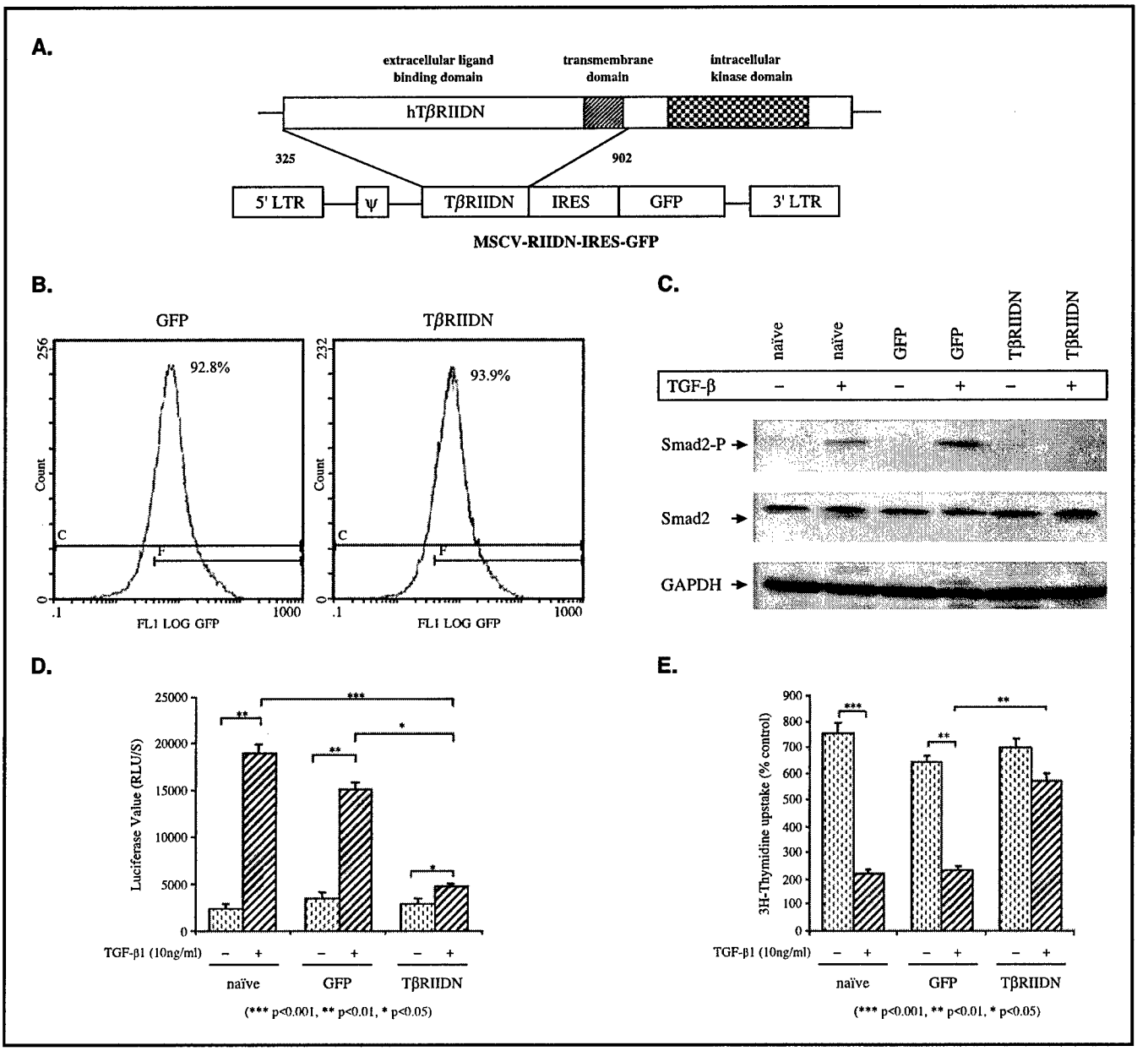


Figure 2. Structure, function, and expression of dominant-negative TβRIIDN. *A,* schematic diagram of the murine stem cell virus (*MSCV*) retroviral construct. A truncated sequence of the human TβRIIDN, lacking the intracellular kinase signaling domain, was cloned into the pMig-internal ribosomal entry sequence (*IRES*)-GFP vector. The control construct (not shown) contained the GFP vector only and without the TβRIIDN sequence (325-902 bp). *B,* fluorescent-activated cell sorting analysis of murine $CD8^+$ T cells transfected with the TβRIIDN vector (93.9%) and the GFP control vector (92.8%). The high efficiency of infection of the viral transgene into $CD8^+$ T cells allowed us to perform adoptive transfer directly without the need of sorting. *C-E,* series of functional analysis done following the treatment of $CD8^+$ T cells with 10 ng/mL TGF-β1. *C,* phosphorylation of Smad-2 observed in naive $CD8^+$ T cells and in tumor-reactive $CD8^+$ T cells infected with the GFP control vector but not observed in tumor-reactive $CD8^+$ T cells infected with the TβRIIDN vector. Blots were stripped and reprobed with anti-Smad-2 and anti-GAPDH antibodies as loading controls. *D,* luciferase activity in $CD8^+$T cells transfected by TGF-β signal-specific plasminogen activator inhibitor-1 promoter-luciferase reporter construct and treated with TGF-β1. *E,* [^3H]-thymidine incorporation into $CD8^+$ T cells before and after the treatment with TGF-β1. *Bars,* SD.

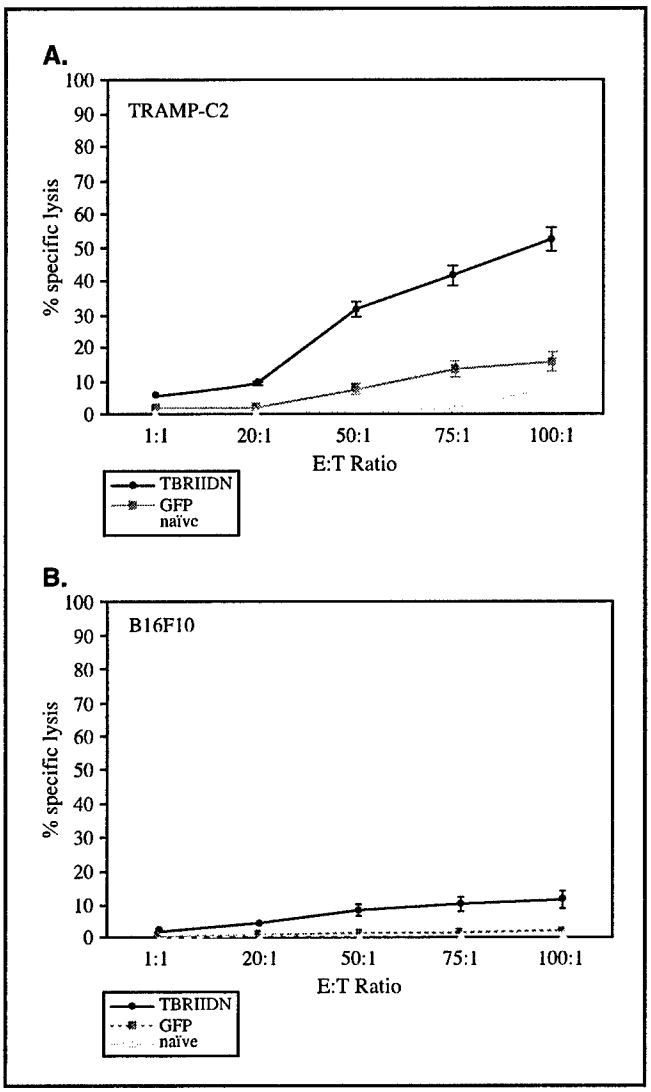
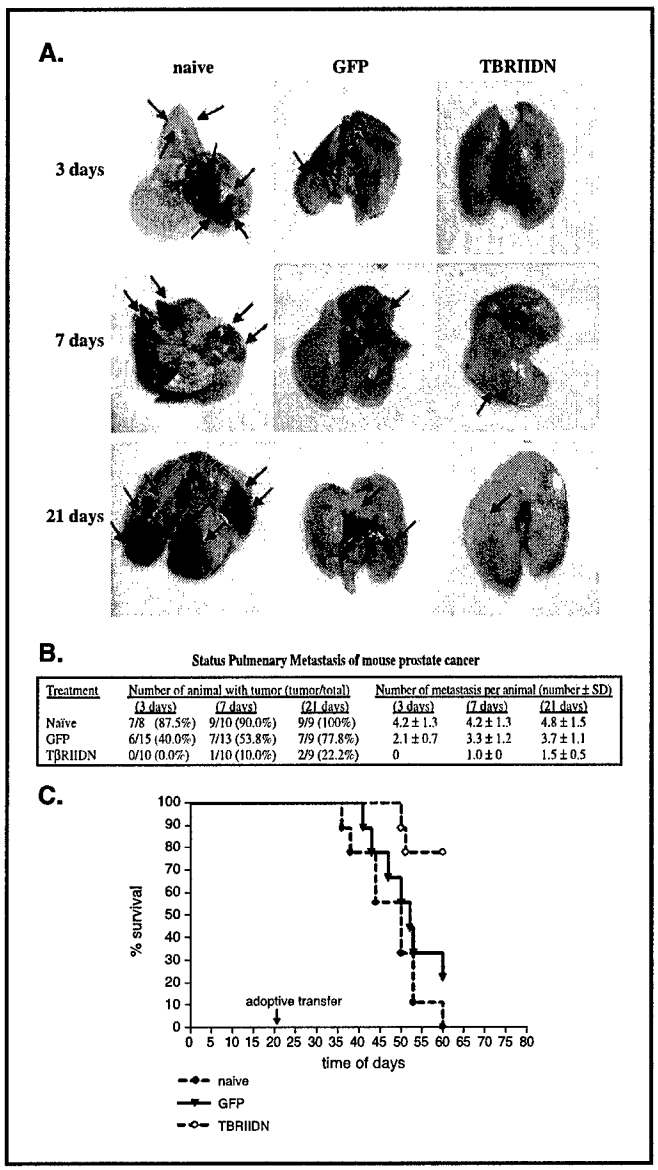


Figure 3. *In vitro* CTL assays. CTL was done using the conventional ^{51}Cr release assay (see Materials and Methods). Naive $CD8^+$ T cells, GFP, and TβRIIDN transfected $CD8^+$ T cells were cocultured with ^{51}Cr-labeled targets at the specified E/T ratios. *A*, TRAMP-C2 mouse prostate cancer cells were used as the targets; *B*, B16-F10 mouse melanoma cells were used as targets. *Point*, average observations obtained from eight wells; *bars*, SD.

(green, 50 μL). The same slides were treated with blocking buffer and probed for CD8 with rat monoclonal antibody, which was labeled with APC (red; 2 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA). Finally, the same slides were stained for cell nuclei with VECTASHIELD mounting media (blue; Vector Laboratories, Burlingame, CA). They were viewed with Nikon TE2000-U fluorescent microscopy (Nikon Co., Tokyo, Japan). Images were digitized by Photoshop 7.0 software.

Expression of TGF-β Receptors in $CD8^+$ T Cells. Normal $CD8^+$ T cells, isolated from freshly harvested spleens, were subjected to fixation and permeabilization in preparation for immunofluorescent staining and flow cytometry analysis. Cells were stained with phycoerytherin-conjugated monoclonal antibody against TGF-β type I receptor (1:100 dilution; Santa Cruz Biotechnology), and TGF-β type II receptor (1:50 dilution, Santa Cruz Biotechnology). They were then stained with FITC-conjugated monoclonal antibody against CD8 (Santa Cruz Biotechnology). These cells were subjected to dual analysis for phycoerytherin and FITC by flow cytometry.

Western Blot Analysis for SMAD-2 Phosphorylation. $CD8^+$ T cells were treated with or without 10 ng/mL of TGF-β1 for 16 hours (19). Cell lysates were prepared by adding radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, 1% NP40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF) to cell pellets. Approximately 30 μg of total protein extract were loaded onto 10%



Treatment	Number of animal with tumor (tumor/total) (3 days)	(7 days)	(21 days)	Number of metastasis per animal (number ± SD) (3 days)	(7 days)	(21 days)
Naïve	7/8 (87.5%)	9/10 (90.0%)	9/9 (100%)	4.2 ± 1.3	4.2 ± 1.3	4.8 ± 1.5
GFP	6/15 (40.0%)	7/13 (53.8%)	7/9 (77.8%)	2.1 ± 0.7	3.3 ± 1.2	3.7 ± 1.1
TβRIIDN	0/10 (0.0%)	1/10 (10.0%)	2/9 (22.2%)	0	1.0 ± 0	1.5 ± 0.5

Figure 4. Status of pulmonary metastasis of TRAMP-C2 tumors in mice received adoptive transfer of three types of $CD8^+$ T cells. Recipient mice received a single injection of TRAMP-C2 cells (5×10^5). At 3, 7, or 21 days following the initial tumor challenge, adoptive transfer of $CD8^+$ T cells was done. Animals were sacrificed at 40 days following the adoptive transfer or sooner due to poor health conditions. *A*, representative gross feature of lung tissues from tumor-bearing mice at 40 days following the administration of adoptive transfer. *Arrows*, metastatic sites. *B*, status of pulmonary metastasis of mouse prostate cancer. The presence of gross and microscopic pulmonary metastasis in each treatment group was tabulated and expressed as the number of animals with pulmonary metastasis (% total animals in each group) as well as the average number of metastatic sites per animal. Results of the statistical analysis, using the χ^2 test, indicated that values in the TβRIIDN group are significantly different from those of other two groups ($P < 0.001$). Also, values in the GFP group are significantly different from those of the naive group ($P < 0.001$). *C*, Kaplan-Meier survival curve of tumor-bearing mice received adoptive transfer of naive $CD8^+$ T cells (*dotted line with solid circles*), GFP control vector (*solid line with solid circles*), and TβRIIDN-transfected $CD8^+$ T cells (*dotted line with open circles*). $P < 0.05$ according to the log-rank test for the TβRIIDN group versus the naive or GFP group.

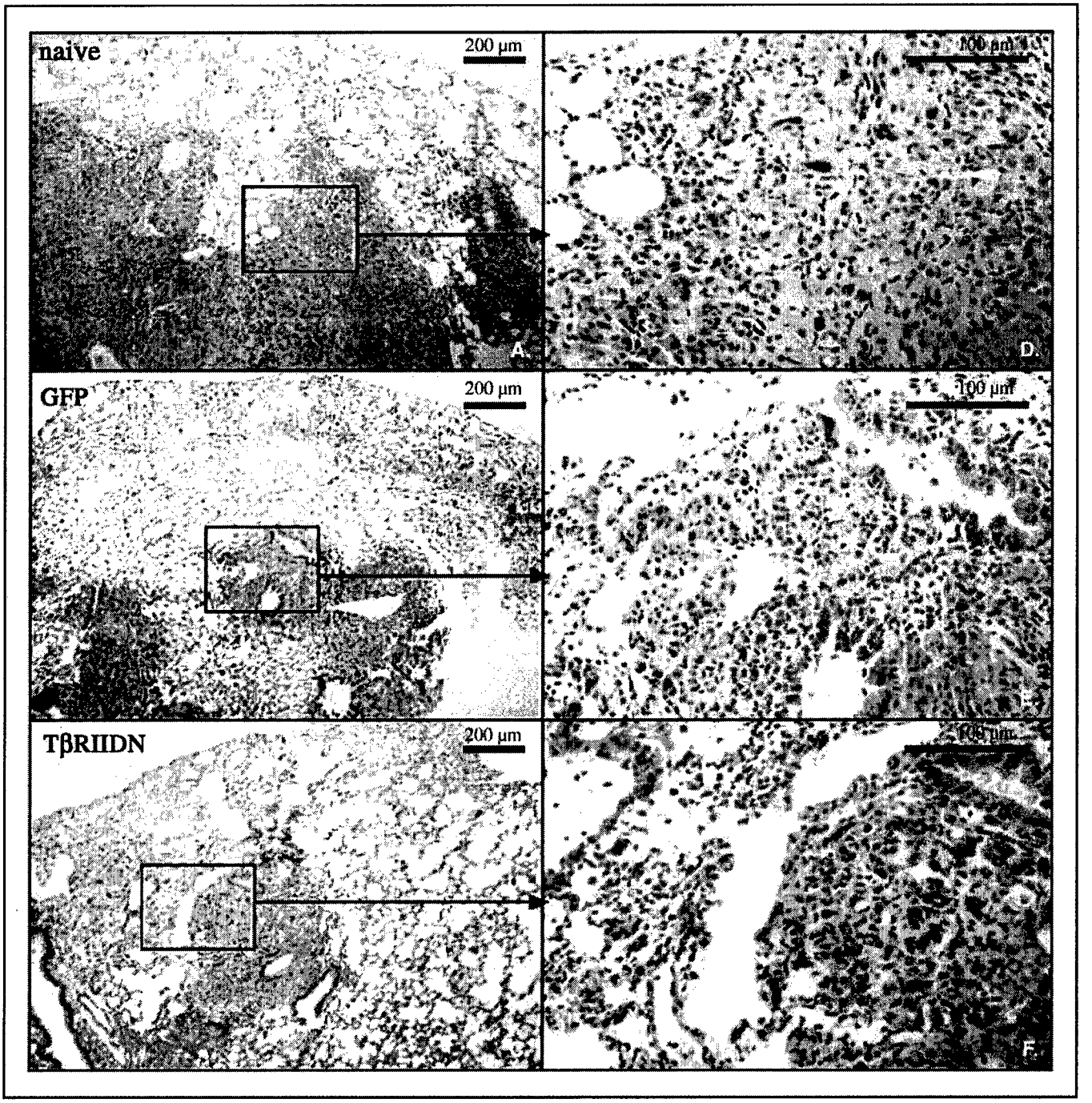


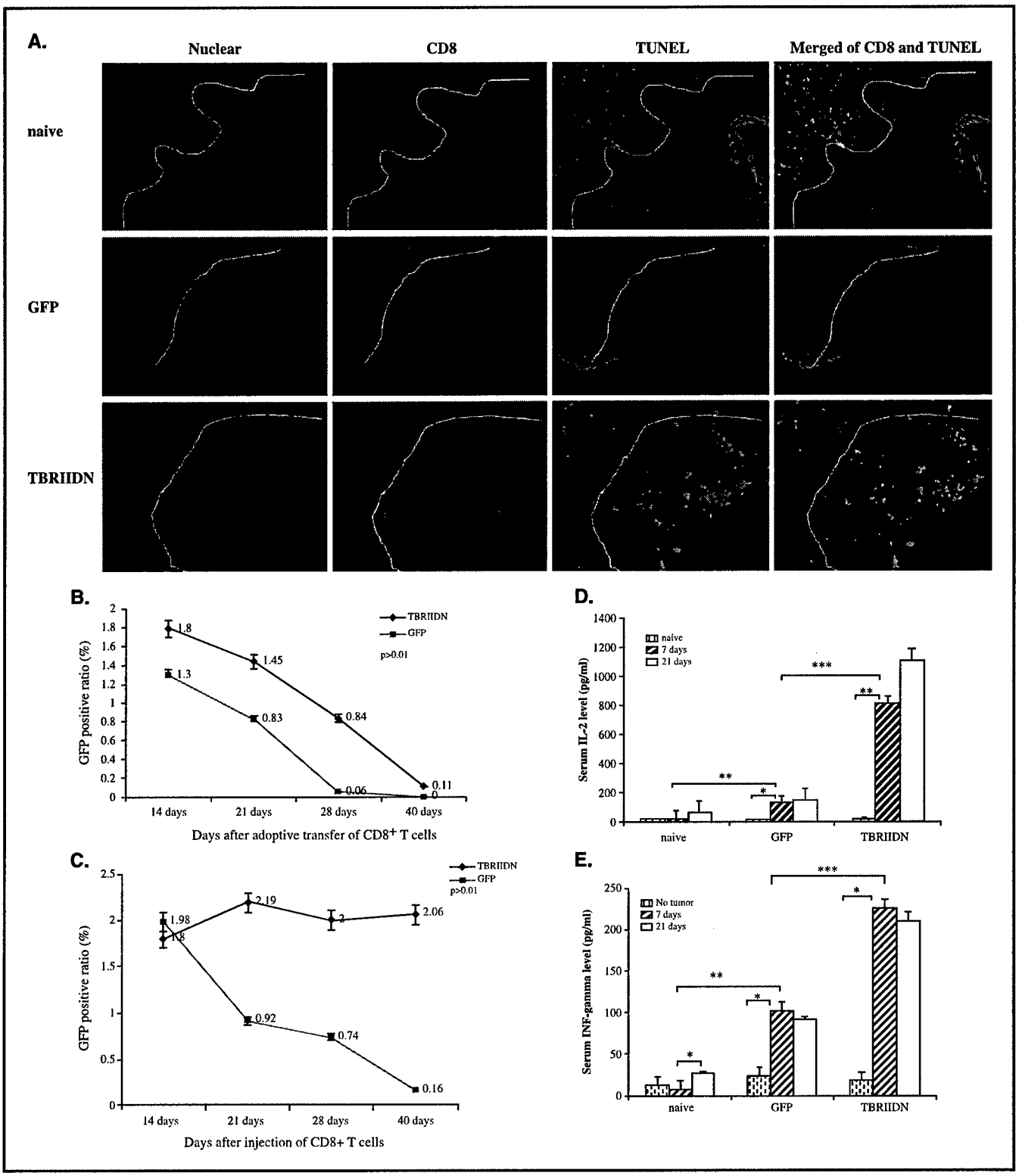
Figure 5. Representative histologic features (H&E staining) of metastatic tumor nodules in the lungs from animals received adoptive transfer of naive $CD8^+$ T cells (*A* and *D*), GFP-infected tumor reactive $CD8^+$ T cells (*B* and *E*), and TβRIIDN-infected tumor reactive $CD8^+$ T cells (*C* and *F*) mice 40 days following the adoptive transfer. These animals received injection of tumor cells 7 days before the adoptive transfer. *A*, lung tissue of a mouse which received adoptive transfer of naive $CD8^+$ T cells showing a portion of a large tumor (4 mm in diameter) with marked cytologic polymorphism (*D*). *B*, lung tissue of a mouse which received adoptive transfer of tumor-reactive control $CD8^+$ T cells, which contained the control GFP vector. There are two smaller tumor nodules (0.5 and 0.6 mm in diameter, respectively), which showed some immune cell infiltration and degenerative changes of tumor cells (*E*). *C*, lung tissue of a mouse which received adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells. There is a smaller tumor nodule (0.5 mm in diameter). Within this tumor, heavy immune cell infiltrates and marked degenerative changes of tumor cells can be seen (*F*). This impression was confirmed in Fig. 6*A*.

Q3 acrylamide gel in Tris-HCl (Bio-Rad, Richmond, CA). Electrophoresis was carried out in Tris-glycine-SDS running buffer and transferred to a polyvinylidene difluoride membrane. Blots were probed for phosphorylated *SMAD-2* with a monoclonal antibody. They were then stripped and reprobed for *SMAD-2* and glyceraldehyde-3-phosphate dehydrogenase. Proteins of interest were detected with the enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom) followed by exposure to Kodak X-OMAT AR film.

Plasminogen Activator Inhibitor-1 Promoter-Reporter Activity Assay. $CD8^+$ T cells were transiently transfected with a promoter construct,

3TP-Lux, which contains multiple copies of TGF-β response element, using LipofectAMINE 2000 (Invitrogen, San Diego, CA). Cells were treated with 10 ng/mL of TGF-β1 for 16 hours. Luciferase activity was assayed by using an assay kit (Promega, San Diego, CA). Activity was normalized based on β-galactosidase expression with pSVβ-galactosidase.

Thymidine Incorporation Assay. $CD8^+$ T cells (3×10^4 cells per 24 wells) were treated with or without TGF-β1 (10 ng/mL) for 16 hours. A medium containing [^{3}H]-thymidine (0.5 μCi/mL; Amersham Biosciences) was introduced and cells were cultured for additional 5 hours. The experiment was terminated by washing with warm serum-free medium.



NaOH (0.1 mol/L) was added to all wells (1 mL). An aliquot of 100 μL was removed for measurement of the protein content and the remainder was used for determining the radioactivity. Thymidine incorporation was expressed as the fraction of counts found in controls.

TGF-β1 ELISA Assay. TRAMP-C2 cells (1.0×10^7 per T75 flask) were cultured in serum-free media for 24 hours. The medium was replaced for 24 hours. The pooled conditioned medium was collected and concentrated by using YM-3 Centriprep Centrifugal Filter Devices (Millipore Co., Bedford, MA). After activation of TGF-β1 by treatment with 1 N HCl (0.1 mL per 0.5mL per conditioned media), the mixture was neutralized by 0.1 mL 1.2 N NaOH/0.5 mol/L HEPES. The ELISA assay was carried out using the Quantikine Human TGF-β1 Immunoassay Kit from R&D Systems (Minneapolis, MN). The total number of cells in each flask was counted using a Coulter Counter and levels of TGF-β1 were reported as pg per 10^5 cells per 48 hours.

Fate of Tumor-Reactive CD8$^+$ T Cells in the Spleen. Because tumor-reactive CD8$^+$ T cells are labeled with GFP, the percent of GFP positive CD8$^+$ T cells in the spleen was determined by flow cytometry. This experiment was carried out in both tumor-free animals and in tumor-bearing animals at different time points.

Statistical Methods. All *in vitro* experiments were done in triplicate. Numerical data were expressed as mean ± SD. ANOVA and multiple range test were done to determine differences of means among different treatment groups. $P < 0.05$ was considered statistically significant. The Q5 SPSS 10.0.7 software package (SPSS, Inc., Chicago, IL) was used for analysis. Kaplan-Meier survival curve was analyzed by the log-rank test using the Graphpad Prism 4.02 software (Graphpad Software, Inc., San Diego, CA).

Results

TGF-β1 Production in TRAMP-C2 Cells

TRAMP-C2 cells secreted 170 pg TGF-β1 in 10^5 cells over 48 hours. For comparison, TGF-β1 production was measured in other murine cell lines (B16, Renca), human cancer cell lines (LNCaP, PC3, and TSU-Pr1) and a benign human prostate epithelial F1 line, RWPE-1 (Fig. 1*A*).

Status of TGF-β Signaling in CD8$^+$ T Cells

Under normal conditions, CD8$^+$ T cells expressed high levels of type I and type II TGF-β receptors (Fig. 1*B*) and they are highly sensitive to the inhibitory effect of TGF-β (12). When CD8$^+$ T cells were infected with the retrovirus containing TβRIIDN, they became insensitive to TGF-β, as shown by following tests. TβRIIDN infected CD8$^+$ T cells were insensitive to TGF-β1 by a lack of SMAD-2 phosphorylation, which was observed in naive F2 CD8$^+$ T cells or cells infected with GFP control vector (Fig. 2*C*). Similarly, there was a significant inhibition of plasminogen activator inhibitor-1 promoter-luciferase reporter activity in TGF-β-insensitive CD8$^+$ T cells in response to TGF-β1 (Fig. 2*D*). Finally, these cells were insensitive to TGF-β1-mediated inhibition of thymidine incorporation assay (Fig. 2*E*).

In vitro Antitumor Activity of Tumor-Reactive TGF-β-Insensitive CD8$^+$ T Cells

Tumor-reactive TGF-β-insensitive CD8$^+$ T cells showed a potent-specific lysis against TRAMP-C2 cells (Fig. 3*A*). These cells showed F3 a 5-fold more tumor-killing activity than that of TGF-β-sensitive counterpart and 25-fold over that of naive CD8$^+$ T cells. Both the TGF-β-sensitive and TGF-β-insensitive tumor-reactive CD8$^+$ T cells showed a reduced tumor-killing activity when incubated with an irrelevant cell line, mouse B16-F10 melanoma cells (Fig. 3*B*).

In vivo Antitumor Activity of Tumor-Reactive TGF-β-Insensitive CD8$^+$ T Cells

In the absence of any intervention, at 21 days following the injection of tumor cells, multiple pulmonary gross and microscopic pulmonary metastases were evident (data not shown). Those animals which received adoptive transfer of tumor-reactive TGF-β-insensitive CD8$^+$ T cells had the least degree of tumor burden (Fig. 4*A* and *B*). There was no evidence of pulmonary F4 metastasis in the group of mice which received adoptive transfer at 3 days following the injection of tumor cells. One of 10 animals in the 7-day group was found to have microscopic evidence of pulmonary metastasis at the time of sacrifice. Two of nine animals in the 21-day group must be sacrificed earlier due to poor health conditions and were found to have pulmonary metastasis of the tumor. Animals which received adoptive transfer of tumor-reactive control CD8$^+$ T cells (GFP only) showed an intermediate degree of tumor burden; whereas those which received adoptive transfer of naive CD8$^+$ T cells were ineffective in inhibiting tumor progression. Analysis of Kaplan-Meier survival cure showed highly significant differences among three treatment groups (Fig. 4*C*).

Histologic Findings

The most prominent histologic feature of the tumor tissue in this study is the evidence of infiltration of CD8$^+$ T cells into the tumor tissue and the presence of apoptosis in tumor cells of animals which received adoptive transfer of tumor-reactive TGF-β-insensitive CD8$^+$ T cells (the TβRIIDN group; Fig. 6*A*). Tumors in animals F6 of the other two groups showed little or no CD8$^+$ T cells and showed no evidence of apoptosis in tumor cells (Fig. 6*A*). CD8$^+$ T cells, however, are present in the parenchymal tissue of the lung in animals of all groups (Fig. 6*A*). Although CD8$^+$ T cells were not observed in tumors of animals in the GFP group, immune cell infiltration was apparent according to histologic observation (Fig. 5*E*), but such immune cell infiltration was more prominent F5 in tumor of the TβRIIDN group (Fig. 5*F*). No immune cell infiltration was noted in tumors of animals which received adoptive transfer of naive CD8$^+$ T cells (Fig. 5*D*).

Another feature is the lack of infiltration of immune cells in the air space of the lung in all animals, including those which received adoptive transfer of tumor-reactive TGF-β-insensitive

Figure 6. *A*, immunofluorescent staining for nuclei, CD8$^+$ T cells, and apoptosis in pulmonary metastasis. Representative tissue sections of pulmonary metastasis from the day 7 group were simultaneously stained for cell nucleus (*blue*), CD8$^+$ T cells (*red*), and apoptosis (*green*). Metastatic sites were identified by the nuclear staining (*blue*). (*blue*). CD8$^+$ T cells (*red*) were identified mainly in the parenchyma of lung tissues not in the tumor with the exception of the TβRIIDN group, in which CD8$^+$ T cells (*red*) were also found within the tumor lesion. Frequent tumor apoptotic sites (*green*) were only found in the TβRIIDN group. Although few CD8$^+$ T cells were found undergoing apoptosis (*yellow*), the majority of the apoptotic cells were derived from the tumor cells (*green*). Magnification, ×40. The fate of adoptively transferred CD8$^+$ T cells in recipients of different treatment groups. A total of 2×10^6 CD8$^+$ T cells were injected via the tail vein into all recipient mice. At designated time intervals, CD8$^+$ T cells from the spleen of each animal were isolated. % GFP-positive CD8$^+$ T cells was calculated by fluorescent-activated cell sorting. *B*, % GFP-positive CD8$^+$T cells in the spleen of tumor-free animals. *C*, % GFP-positive CD8$^+$ T cells in the spleen of tumor-bearing animals. A total of 2×10^6 CD8$^+$ T cells were tail vein injected into tumor-bearing C57BL/6 mice. % GFP-positive CD8$^+$ T cells in the spleen was analyzed by fluorescent-activated cell sorting. *Bars*, SD. *D*, circulating levels of IL-2 and IFN-γ in experimental mice. Serum specimens were collected at the time of animals' euthanasia, which is 40 days following the adoptive transfer of CD8$^+$ T cells. Serum level of IL-2 in different groups of mice. *E*, Serum level of IFN-γ in different groups of mice. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. *Bars*, SD.

$CD8^+$ T cells (Fig. 5). This is in sharp contrast to our earlier studies, in which recipient animals which received TGF-β-insensitive bone marrow transplant and developed massive infiltration of immune cells in the air space of the lung (19, 20). In the present study, the air space was devoid of any immune cells, suggesting that autoimmune disease was not apparent in these animals.

Fate of Transferred $CD8^+$ T Cells

In the present study, a total of 2×10^6 $CD8^+$ T cells were transferred into each recipient animal. This number was derived by extrapolating the comparable number of T cells in adoptive therapy for cancer patients (1, 6). In a similar study with adoptive transfer of experimental melanoma model, 3×10^6 antigen-specific T cells were used (21). To determine the fate of these transferred cells, we measured the percentage of GFP-positive $CD8^+$ T cells in the spleen of recipient animals. When $CD8^+$ T cells were adoptively transferred to tumor-free hosts, there was a linear decay in GFP-positive $CD8^+$ T cells reaching 0% by 30 days for tumor-reactive $CD8^+$ T cells infected with the GFP control vector and 50 days for tumor-reactive $CD8^+$ T cells infected with the TβRIIDN vector (Fig. 6*B*). However, when tumor-reactive TGF-β-insensitive $CD8^+$ T cells were adoptively transferred to tumor-bearing hosts (the TβRIIDN group), the percentage of GFP-positive $CD8^+$ T cells was maintained at 2% for at least 40 days; whereas in animals which received adoptive transfer of tumor-reactive control $CD8^+$ T cells (the GFP group), GFP-positive $CD8^+$ T cells showed a decay curve similar to that in tumor-free hosts with a slight delay (Fig. 6*C*).

Serum Levels of IFN-γ and IL-2

In animals which received adoptive transfer of naive $CD8^+$ T cells, there was a baseline level of IL-2 and IFN-γ. In animals which received tumor-reactive control $CD8^+$ T cells (the GFP group), there was a significant increase in both cytokines. A further increase in serum IL-2 (Fig. 6*D*) and IFN-γ (Fig. 6*E*) was observed when these cells were rendered insensitive to TGF-β (the TβRIIDN group), suggesting the presence of activated immune cells in these hosts.

Discussion

Immunotherapy using adoptive transfer of immune cells is a promising approach for treatment of cancer patients. However, currently available therapies have not achieved a significant number of complete responders. A successful adoptive therapy for cancer should be the development of robust effector cells with specific antitumor efficacy. At the same time, the treatment will overcome the tumor-derived immunosuppressive effect.

A significant part of tumor immunology has focused on the identification of tumor-specific antigens and the cytolytic T cells specific for these peptides (22). Adoptive T-cell therapy using antigen-specific $CD8^+$ T cells for cancer treatment has been attempted with some degree of success (5, 23) and seems to be an advantage over the transfer of nonspecific T cells (21). Results of the present study have shown that adoptive transfer of tumor-reactive and TGF-β-insensitive $CD8^+$ T cells were able to specifically target against autologous tumor cells and eradicate established pulmonary metastasis. The use of tumor-specific adoptive immunotherapy has reported before and has clearly shown its efficacy by other investigators (23, 24). The critical issue in immunotherapy thus far has been the tumor-derived immunosuppressive effect, which remains unresolved.

The mouse prostate cancer model, TRAMP-C2, represents an aggressive line of malignant cells, which secrete large amounts of TGF-β. The role of immunosuppressive effect of TGF-β in cancer progression has been well established (13, 25–27). In the present study, we have shown that, TRAMP-C2 tumors possess potent immunosuppressive power so that regular $CD8^+$ T cells are unable to infiltrate into the tumor tissues. However, if these tumor-reactive $CD8^+$ T cells are engineered and rendered insensitive to TGF-β, they are able to infiltrate into the tumor tissue and induce apoptosis in these established TRAMP-C2 tumors. To the best of our knowledge, studies to test this concept have not been attempted before. These results support the concept that TGF-β is an important target in cancer therapy.

Results of the present study show that in tumor-bearing hosts, the transferred $CD8^+$ T cells persist, only if they are tumor reactive and TGF-β insensitive. Therefore, adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells will persist in tumor-bearing hosts and does not require the procedure of lymphodepletion. Interestingly, these cells decayed in tumor-free hosts. Accompanied with the persistence of these transferred $CD8^+$ T cells were elevated circulating levels of IL-2 and IFN-γ, a critical requirement for antitumor activity in the host (24). Therefore, with our current approach of adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells, exogenous treatment of IL-2 is not necessary for a successful antitumor activity. On the other hand, naive $CD8^+$ T cells and tumor-reactive $CD8^+$ T cells but sensitive to TGF-β did not persist in the host, suggesting that these cells failed to establish an engraftment regardless the status of the presence or absence of tumor cells in the host. These observations suggest that a single transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells is sufficient for tumor rejection.

Our results also indicate that $CD8^+$ T cells contain high levels of TGF-β receptor types I and II and therefore, are highly sensitive to the inhibitory effects of TGF-β. The role of TGF-β in the immune system is best shown in TGF-β knockout animals. Mice lacking TGF-β, although they grew normally for the first 2 weeks, develop rapid wasting syndrome, and die by 3 to 4 weeks of age (28, 29). These studies showed a powerful immunoregulatory role of TGF-β because $TGF\text{-}\beta^{-/-}$ mice had excessive inflammatory responses with massive infiltration of lymphocytes and macrophages in multiple organs. These syndromes were characterized as autoimmunity (17, 29). Results of our past study have shown that mice receiving TGF-β-insensitive bone marrow transplants have met with the same fate by developing autoimmune syndrome, although these animals were able to eliminate challenged tumors (19, 20). In the present study, the use of the tumor-specific TGF-β-insensitive $CD8^+$ T cells for the treatment of established cancer did not result in the development of massive infiltration of immune cells into the airspace of the lung of tumor-bearing host and, in tumor-free hosts, these $CD8^+$ T cells failed to persist in the host. These preliminary observations seem to suggest an apparent absence of the development of autoimmune disease in these animals. Further studies are warranted to verify this impression.

In summary, the present results showed that adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells to tumor-bearing hosts was able to eradicate autologous tumors. These $CD8^+$ T cells have the following characteristic properties. First, they are specifically reactive against tumor tissues. Second, they are insensitive to TGF-β. These two properties endowed these $CD8^+$

T cells with the ability to infiltrate into tumor tissues and function as potent effectors against tumor cells. Finally, these cells are able to persist in tumor-bearing hosts but not in tumor-free hosts. These findings provide a proof of principle that an adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells may warrant consideration for the treatment of advanced cancers.

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